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Effect of ultrasonic pretreatment on whey protein hydrolysis by alcalase: Thermodynamic parameters, physicochemical properties and bioactivities

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ABSTRACT

The effects of ultrasonic pretreatment on the enzymolysis thermodynamics and physicochemical properties of whey protein, and the mechanisms behind those effects, were investigated. Changes in the angiotensin-I converting enzyme (ACE) inhibitory and immunomodulatory activities of whey protein hydrolysates after ultrasonic pretreatment were also determined. Results showed that the ultrasonicated whey protein had a higher in degree of hydrolysis than the non-sonicated protein. After the pretreatment, the activation energy (E_a), enthalpy of activation (ΔH) and entropy of activation (ΔS) of whey protein enzymolysis were decreased by 15.9%, 16.8%, and 16.4%, respectively. There was no significant change in free energy (ΔG) ($P > 0.05$). Physicochemical analysis revealed that ultrasound had induced unfolding of the whey protein, resulting in a 43.7% increase in its surface free sulfhydryl content and a 62.6% increase in surface hydrophobicity. Ultrasound significantly decreased the protein's α-helical content and significantly increased its β-sheets and β-turns ($P < 0.05$). The ACE inhibitory and immunomodulatory activities of the whey protein hydrolysates were significantly increased by ultrasonic pretreatment ($P < 0.05$). These results suggest that ultrasound can be applied to enhance whey protein enzymolysis for the generation of novel bioactive peptides that can be used as drug or functional food ingredient.

1. Introduction

Ultrasound is a sound wave with a frequency above the range of human hearing; that is, above 20 kHz [1]. It can be classified into two fields on the basis of the frequency range: low-energy ultrasound with a frequency in the range of 100 kHz to 1 MHz, and high-energy power ultrasound with a frequency in the range of 20–100 kHz [2]. Compared with low-energy ultrasound, which is used for analyzing and evaluating the physicochemical properties of foods [2,3], high-energy power ultrasound is widely applied to alter the physicochemical properties of foods in various areas [4]. High-energy power ultrasound can cause variable alterations to food structures depending on the ultrasonic cavitation, the rapid formation and collapse of gas bubbles, which can produce high shear and mechanical energy [3]. Recently, high-energy power ultrasound has been used successfully to improve the enzymatic hydrolysis and properties of proteins. Resendiz-Vazquez et al. [5] found that the application of ultrasound at 20 kHz and 400 W increased the emulsifying activity and emulsion stability of a jackfruit seed protein isolate as well as changed the molecular weight of the protein fraction. Wang et al. [4] reported that ultrasonic pretreatment at 20 kHz significantly increased the degree of hydrolysis (DH) of β-conglycinin (7S) and glycinin (11S) and the antioxidative activity of their hydrolysates. Similarly, Wang et al. [6] reported that ultrasonic pretreatment increased both the hydrolysis rate of oat protein hydrolysates and their angiotensin-I converting enzyme (ACE) activity. The changes in the yield and bioactivities of protein hydrolysates after ultrasound treatment were attributed to the molecular unfolding of the protein and exposure of its functional groups [7,8]. As a result, ultrasonic pretreatment offers a potential way of producing bioactive peptides from proteins. However, the mechanisms behind the effects of ultrasound on protein hydrolysis need to be studied further.

Whey protein, an abundant by-product of the dairy industry,

Abbreviations: DH, degree of hydrolysis; ACE, angiotensin-I converting enzyme; DTNB, 5,5′-Dithiobis(2-nitrobenzoic acid); ANS, 1-anilino-8-naphthalene sulfonate; HHL, Hippuryl-His-Leu; MTT, 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide; DMSO, dimethyl sulfoxide; FBS, Fetal bovine serum; k_{in}, effective (total) rate constant; A, pre-exponential or collision factor; R, universal gas constant; T, Kelvin temperature; k_B, Boltzmann constant; h, Planck constant; E_a, activation energy; Δ, SSentropy of activation; ΔG, free energies of activation; ΔH, enthalpy of activation; SH, sulfhydryl

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consists mainly of β-lactoglobulin (55%–60%, w/w), α-lactalbumin (15%–20%, w/w), and bovine serum albumin (5%–10%, w/w) [9]. It has been widely applied in many protein-based food formulations, primarily attributed to its high nutritional value and desirable functional properties [10]. In recent years, many research studies have confirmed the importance of whey protein as a source of bioactive peptides and the association of their hydrolysates with important biological activities. For example, antioxidative and ACE inhibitory and immunomodulatory activities were observed in the hydrolysates obtained from whey protein using alcalase [11–13]. Although whey protein is rich in bioactive peptides, it is not easily broken down by proteases [14]. Therefore, some studies have focused on the ultrasonic pretreatment method to enhance the enzymatic hydrolysis process [14]. However, the literature about the application of ultrasonic pretreatment to improve whey protein hydrolysis is very limited. Thus, the main objective of this study was to determine the effects of ultrasonic pretreatment on the physicochemical properties of whey protein, its enzymolysis thermodynamics, and the hydrolysate bioactivity. This research should contribute to our further study on the mechanism of ultrasound-accelerated enzymatic hydrolysis of whey protein.

2. Materials and methods

2.1. Materials and chemicals

Whey protein powder (86.5% protein content) manufactured from cow milk, which consisted mainly of β-lactoglobulin and α-lactalbumin (α-lactalbumin- to- β-lactoglobulin ratio of 1: 5, w/w), was purchased from Huangchao Chemical Products Co. (Zhengzhou, China). Alcalase was purchased from Novozymes (China) Biotechnology Co. 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB), 1-anilino-8-naphthalene sulfonate (ANS), ACE (from rabbit lung), Hippuryl-His-Leu (HHL), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Trading Co. (Shanghai, China). Fetal bovine serum (FBS) was purchased from Gibico (Grand Island, NY, USA). Other reagents were of analytical grade.

2.2. Test of the effect of ultrasonic pretreatment on the degree of hydrolysis (DH) of whey protein

2.2.1. Ultrasonic pretreatment of whey protein

Ultrasonic pretreatment was carried out in an ultrasonic processor (JY92-II, Haishukesheng Ultrasonic Equipment Co., Ningbo, China) with a 1.5 cm flat tip probe (20 kHz). Whey protein (10 g) was suspended in 200 ml of deionized water and mixed with a magnetic stirrer. Then the suspension was put into a 250 ml beaker, and the beaker was kept in an ice bath. The sonication was done at levels of power of 0 W (control), 100 W, 200 W, 300 W, 400 W and 500W for 15 min (pulse durations of 2 s on and 2 s off), respectively. The temperature of the protein solution was observed during ultrasound to ensure temperature below 25 °C. After ultrasonic pretreatment, the treated protein solution was then used to analyze the effect of ultrasound power on the degree of hydrolysis (DH) of whey protein. According to the changes of DH, an optimal power was chosen. Then 200 ml of whey protein solution at a concentration of 5% w/v was treated at above selected ultrasonic power for different time (0, 5, 10, 15, 20, 25 min). Then, the treated protein solution was used to analyze the effect of ultrasound time on DH of whey protein.

2.2.2. Whey protein hydrolysis and determination of DH

In this study, the untreated and treated whey proteins were hydrolysed by alcalase (enzyme to substrate ratio, 5000 U/g of protein) at substrate concentrations of 10 g/L. The other enzymolysis conditions were: hydrolysis temperature 55 °C, pH 8.0, hydrolysis time 30 min. The hydrolysis conditions were chosen according to our previous experiments.

The DH was determined using the pH-stat method [8].

$$DH\,(\%) = \frac{BN_b}{\alpha M_p h_{tot}} \times 100 \tag{1}$$

where B is the base consumption, N_b is the base normality, α is the average degree of dissociation of the α-NH_2 groups in the protein substrate, M_p is the mass of hydrolyzed protein, and h_{tot} is the total number of peptide bonds in the protein substrate (9.05 mmol/g protein).

2.3. Measurement of the effect of ultrasonic pretreatment on the thermodynamics of whey protein hydrolysis by alcalase

2.3.1. Ultrasonic pretreatment test

An aliquot (200 ml) of whey protein solution (10 g/L) was treated with ultrasound at 20 kHz at 300 W for 15 min (pulse durations of 2 s on and 2 s off). In this study, the conditions of ultrasonic pretreatment were chosen according to above single factor experiment. After ultrasonic pretreatment, the treated whey protein was used to analyze thermodynamics of hydrolysis.

2.3.2. Determination of thermodynamics parameters

A 200 ml volume of untreated or ultrasound-pretreated whey protein solution (10 g/L) was adjusted to pH 8.0 and hydrolysed using alcalase (enzyme- to- substrate ratio, 5000 U/g of protein) at the temperatures of 20–50 °C, respectively.

The alcalase hydrolysis of whey protein can be simply described by the first-order kinetic equation, according to Ma et al. [15]:

$$\ln C = -k_{in} t + lnC_0 \tag{2}$$

where t is the hydrolysis time, C_0 is the initial whey protein concentration, C is the whey protein concentration at a determined time t, and k_{in} is the effective (total) rate constant. As it is difficult to measure the decrement of whey protein directly, the reaction rate can be reflected by the decreased amount of peptide bonds in the protein. The total number of peptide bonds (h_{tot}) was estimated to be 9.05 mmol/g, as determined from the amino acid composition [16]. The decreased amount of peptide bonds (h_{dec}) was calculated as a function of the DH value [8]:

$$h_{dec} = h_{tot} \times DH \tag{3}$$

The observed value of k_{in} can be determined by Eq. (4):

$$\ln C_{pb} = -k_{in} t + lnC_{tpb} \tag{4}$$

where C_{pb} is the concentration of peptide bonds in the whey protein at time $t = t$; C_{tpb} is the total concentration of peptide bonds in the whey protein, which is based on the value of h_{tot} (9.05 mmol/g protein).

The temperature dependence of the rate constant k_{in} can be described by Arrhenius equation:

$$k_{in} = Ae^{\frac{-E_a}{RT}} \tag{5}$$

where A is pre-exponential or collision factor, Ea is the activation energy, R is the universal gas constant (8.314 J/mol K), T is the Kelvin temperature. Eq. (5) is solved to give the logarithmic equation,

$$\ln k_{in} = \ln A - \frac{E_a}{RT} \tag{6}$$

Eq. (6) is used to calculate E_a and A by plotting $\ln k_{in}$ versus $1/T$. The plot of $\ln k_{in}$ versus $1/T$ shall give a straight line. The slope and intercept are $-E_a/R$ and $\ln A$ respectively, from which E_a and A can be calculated.

Thermodynamic parameters for alcalase hydrolysis of whey protein were estimated using the Eyring transition state theory, as shown in Eq. (7):